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Distribution of two C cycle enzymes in soil aggregates of a prairie chronosequence

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Abstract Knowledge of the cycling and compartmentalization of soil C that influence C storage may lead to the development of strategies to increase soil C storage potentials. The objective of this study was to use soil hydrolases and soil aggregate fractionation to explore the relationship between C cycling activity and soil aggregate structure. The prairie chronosequence soils were native prairie (NP) and agricultural (AG) and tallgrass prairies restored from agriculture in 1979 (RP-79) and 1993 (RP-93). Assays for β-glucosidase (E.C. 3.2.1.21) and *N*-acetyl-β-glucosaminidase (NAGase, EC 3.2.1.30) activities were conducted on four aggregate size fractions (>2 mm, 1–2 mm, 250 μm–1 mm, and 2–250 μm) from each soil. There were significantly greater amounts of >2-mm aggregates in the RP-79 and RP-93 soils compared to the NP and AG soils due to rapid C accumulation from native plant establishment. Activities for both enzymes (μg PNP g^{-1} soil h^{-1}) were greatest in the microaggregate (2–250 μm) compared to the macroaggregate (>2 mm) fraction; however, microaggregates are a small proportion of each soil (<12%) compared to the macroaggregates (~75%). The RP soils have a hierarchical aggregate system with most of the enzyme activity in the largest aggregate fractions. The NP and AG soils show no hierarchical structure based on aggregate C accretion and significant C enzyme activity in smaller aggregates. The distribution of enzyme activity may play a role in the storage of C whereby the aggrading restored soils may be more susceptible to C loss during turnover of macroaggregates compared to the AG and NP soils with less macroaggregates.

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Introduction

Soils are a source and a sink of greenhouse gases, including N_2O, CH_4, and CO_2; however, recent attention has focused on soils as a potential sink for atmospheric CO_2 (Metting et al. 2001) Thus, detailed investigations of mechanisms of soil C sequestration are necessary to discover potential ways to reduce the rising levels of greenhouse gases. It is hypothesized that through management of soils, such as no-tillage in agricultural systems, an increase in soil C can occur until a maximum capacity is reached (Smith 1994). Increasing soil C is beneficial; however, where the increased C is stored will be important for determining the long-term sequestration potential. Labile organic C, from plant residue decomposition, that resides in large aggregates may be susceptible to rapid decomposition upon disturbance (Elliott 1986), whereas more resistant C compounds associated with small aggregates may persist longer. Ultimately, soil microflora, and the associated soil enzymes, will determine the fate of C residing in aggregates and the loss and stabilization of particular C compounds.

Soil aggregates consist of a hierarchy of decaying SOM and soil mineral particles of clay, sand, and silt. Microaggregates contain small pieces of recalcitrant SOM which are physically protected from decomposition by mineral soil particles that accumulate on and around the surface of the SOM (Cambardella and Elliott 1994). Macroaggregate size particles consist of more labile particulate organic matter (POM) C which may account for about 40% of the total soil organic C. Microbial exudates, fungal hyphae, and POM combine to form a glue that holds microaggregates into larger macroaggregate structures (Tisdall and Oades 1982; Jastrow and Miller 1998). The rate of C turnover in macroaggregates is faster than in microaggregates as indicated by the greater amount of freshly added C incorporated into macroaggregates than into microaggregates (Jastrow 1996; Six et al. 2001). Thus, macroaggregate

turnover is a critical component of soil C stabilization (Six et al. 2004).

It is well recognized and documented that soil enzyme assays are difficult to interpret with respect to activities occurring in situ (Nannipieri et al. 1990, 2002; Dick 1992); however, the activities of enzymes produced by microorganisms are potentially sensitive indicators of change in the biochemical composition of the SOM or of C content in the soil (Deng and Tabatabai 1996; Bandick and Dick 1999; Ekenler and Tabatabai 2003). Soil hydrolase activity governs the flow and fate of C substrates to microorganisms and is thus a controlling factor in C storage potential. The activity of glycosidases, such as β-glucosidase, is significantly correlated to the C content of a soil since these enzymes are instrumental in the breakdown of cellulose and are synthesized by both bacteria and fungi (Deng and Tabatabai 1996). Soil polysaccharide chitin is hydrolyzed by *N*-acetyl glucosaminidase (NAGase, EC 3.2.1.52) and yields glucosamine, making NAGase an important enzyme in C and N cycling (Guggenberger et al. 1999). NAGase activity is strongly associated with fungal-dominated systems. Miller et al. (1998) compared the activity of a diverse group of fungi to that of chitinolytic bacteria and actinomycetes and determined that only the fungi displayed constitutive NAGase activity.

The objectives of this study were to investigate the relationship between the activity of two C cycling enzymes, β-glucosidase and NAGase, and soil aggregate structure by assessing the enzyme activity for aggregate fractions across a chronosequence of similar soil types and vegetation. By identifying physical locations within the soil structure that display the greatest amount of biochemical activity across a C aggrading chronosequence, we can develop an understanding of how aggregate protection and C sequestration are related.

Materials and methods

Soil sampling

The tallgrass prairie restoration chronosequence at Fermi National Laboratory (41.8°N and 88.3°W, Batavia, IL, USA) was sampled during the spring of 2003. The site has a mean annual precipitation of 920 mm and a mean annual temperature of 11°C (Jastrow 1987). The soils (located within a 2.41-km radius) were collected from a remnant native prairie (NP), agricultural land that was restored to prairie grassland in 1979 and 1993 (RP-79 and RP-93, respectively), and agricultural land (AG) that continues to be in a long-term rotation of row crops, maize (*Zea mays*), and beans (*Phaseolus vulgaris*). The soil in the Drummer series was classified as a fine silty, mixed, superactive, mesic Typic Endoaquoll; selected characteristics are presented in Table 1. Soil from each plot was sampled to a depth of 5 cm, passed through a 4-mm sieve, visible plant debris was removed by hand, and the soil was stored at 4°C until analysis.

Table 1 Selected soil characteristics

Plot	pH	Organic C (mg g^{-1})	Total N (mg g^{-1})	F/B[a]
Native prairie (NP)	7.5	118	8.9	ND[b]
Currently farmed (AG)	5.6	41	3.1	0.9
Restored prairie (RP-93)	6.6	59	5.6	10.7
Restored prairie (RP-79)	7.3	60	5.1	13.5

[a]F/B fungal to bacteria ratio (Bailey et al. 2002)
[b]*ND* Not determined

Wet sieving

The wet sieving method for aggregate distribution used in our experiment(s) was adapted from those of Jastrow (1987) and Angers and Mehuys (1993). The sieve sizes were 2 mm, 1 mm, 250 μm, and 53 μm; however, the size fractions collected were >2 mm, 1–2 mm, 250 μm–1 mm, and 2–250 μm. The 53-μm sieve was used to preserve microaggregate structure and limit the amount of material to recover from centrifugation (see below). The sieves were nested with the largest mesh on top and placed into a large basin. Distilled water was added to the basin until the water level reached 1 cm below the wire mesh of the 2-mm sieve. The soil (100 g dry wt) was spread evenly over the surface of the 2-mm mesh, and the water level was raised just until the soil could be wet by capillarity. The soil was allowed to moisten for 10 min, and then the water level was raised to 1.5 cm above the mesh of the top sieve. Sieving consisted of raising and lowering the nest of sieves 4 cm at a rate of 50 times/min for 2 min.

After sieving, the soil remained in the basin undisturbed for 5 min to allow fine particles to settle. Floating organic matter was aspirated into a vacuum flask and rinsed onto a 20-μm nylon filter. The nest of sieves was slowly removed from the basin and placed onto a catch pan to collect any remaining water. The sieves were separated and placed into a 27°C forced air oven and air dried until the aggregates could easily be removed from the sieve. The aggregates were air dried to −0.033 MPa water content (pressure plate method) (Klute 1986).

Water from the basin and catch pan were combined and centrifuged in a Beckman J2-21 centrifuge at 14,300×*g* for 30 min to collect particles greater than 2 μm that were not retained on the 250-μm sieve (the 2-mm to 250-μm fraction). Stoke's law was used to determine the force and time required for the particles to be removed from the solution. After centrifuging, the soil particles from this fraction were combined in a preweighed container and allowed to dry to −0.033 MPa water content in a forced air oven at 27°C. Three macroaggregate size fractions (>2 mm, 1–2 mm, 250 μm–1 mm) and one microaggregate fraction (2–250 μm) were collected.

All of the soil fractions were incubated at the −0.033 MPa water content for 3 days before further analyses to minimize skewing of metabolic activity caused by handling the soil.

Enzyme assays

Assays of β-glucosidase (EC 3.2.1.21) and *N*-acetyl-β-glucosaminidase (NAGase, EC 3.2.1.30) activities were performed on whole soil and all aggregate size fractions from the four soils of the chronosequence. The protocol for the *N*-acetyl-β-glucosaminidase assay was conducted according to Parham and Deng (2000), and β-glucosidase was assayed as described by Eivazi and Tabatabai (1988). Enzyme activity is reported per gram of aggregate size fraction. Preliminary experiments were evaluated for the effect of water saturation on enzyme activity. Both enzymes showed little change (<10%) in activity due to saturation with water. Changes in activity due to disturbance were minimized by a preincubation period of 3 days at −0.033 MPa moisture tension.

Statistical analysis

The data were analyzed using Systat 10 (SPSS Inc., Chicago, IL). For all multiple comparisons, Bonferroni's pairwise comparison test was used, following a one-way ANOVA, to detect significant differences at $P<0.05$. The enzyme activity data were grouped and analyzed by (1) comparing the activity of an enzyme in the different size fractions of each soil and (2) comparing the activity of an enzyme in one size fraction across all four soils. Reported R^2 values represent Pearson's correlation coefficients.

Results

Aggregates

The native prairie (NP) and agricultural (AG) soils had a similar aggregate size distribution (Table 2). The younger restored prairie soil (RP-93) had about 66% more mass in large macroaggregates (>2 mm) than both the NP and AG soils, accompanied by a small proportion of 1–2 mm, 250 μm–1 mm, and 2–250 μm macro- and microaggregates. In the 24-year-old restored prairie soil (RP-79), more of the mass is distributed into the 1–2 mm and 250 μm–1 mm macroaggregate size fractions compared to the RP-93 soil. Like the RP-93 soil, the RP-79 soil had greater than 40% more macroaggregates (1–2 and >2 mm) than both the AG and NP soils.

β-Glucosidase activity

The β-glucosidase activity in the NP soil and the restored prairie (RP-79 and RP-93) soils showed similar distributions through aggregate size fractions (Fig. 1a). However, there was a significant positive correlation ($R^2=0.95$, $P<0.03$) between enzyme activity and percent aggregate fraction in the AG soil from >2 mm to 2–250 μm (Fig. 1a).

β-Glucosidase activity was significantly greater in the smallest size fraction (2–250 μm) for all chronosequence soils, except in the AG soil (Fig. 1a). The trend exhibited by

Table 2 Aggregate size fraction distribution (%) and the organic carbon and total nitrogen content of each fraction across the soils of the prairie chronosequence

Soil	Aggregate size fraction	Aggregate size fraction % of total[a]	mg C g^{-1} soil in each aggregate size fraction	mg N g^{-1} soil in each aggregate size fraction
Native prairie (NP)	>2 mm	27a	24.49	1.86
	1–2 mm	34b	39.40	3.14
	250 μm–1 mm	33a	43.53	3.48
	2–250 μm	7c	5.87	0.48
	Total	100	113.29	8.97
Agriculture (AG)	>2 mm	25a	10.36	0.77
	1–2 mm	30a	12.14	0.91
	250 μm–1 mm	34a	14.44	1.07
	2–250 μm	11b	4.14	0.30
	Total	100	41.08	3.06
1993 restored prairie (RP-93)	>2 mm	74a	40.38	2.85
	1–2 mm	13b	7.71	0.56
	250 μm–1 mm	8c	4.59	0.32
	2–250 μm	5c	2.90	0.21
	Total	100	55.57	3.94
1979 restored prairie (RP-79)	>2 mm	52a	30.83	2.45
	1–2 mm	27b	16.40	1.28
	250 μm–1 mm	17b	11.16	0.85
	2–250 μm	4c	1.85	0.14
	Total	100	60.24	4.72

[a]Within soil aggregate size differences, same letter are not significantly different (Bonferroni, $P<0.05$)

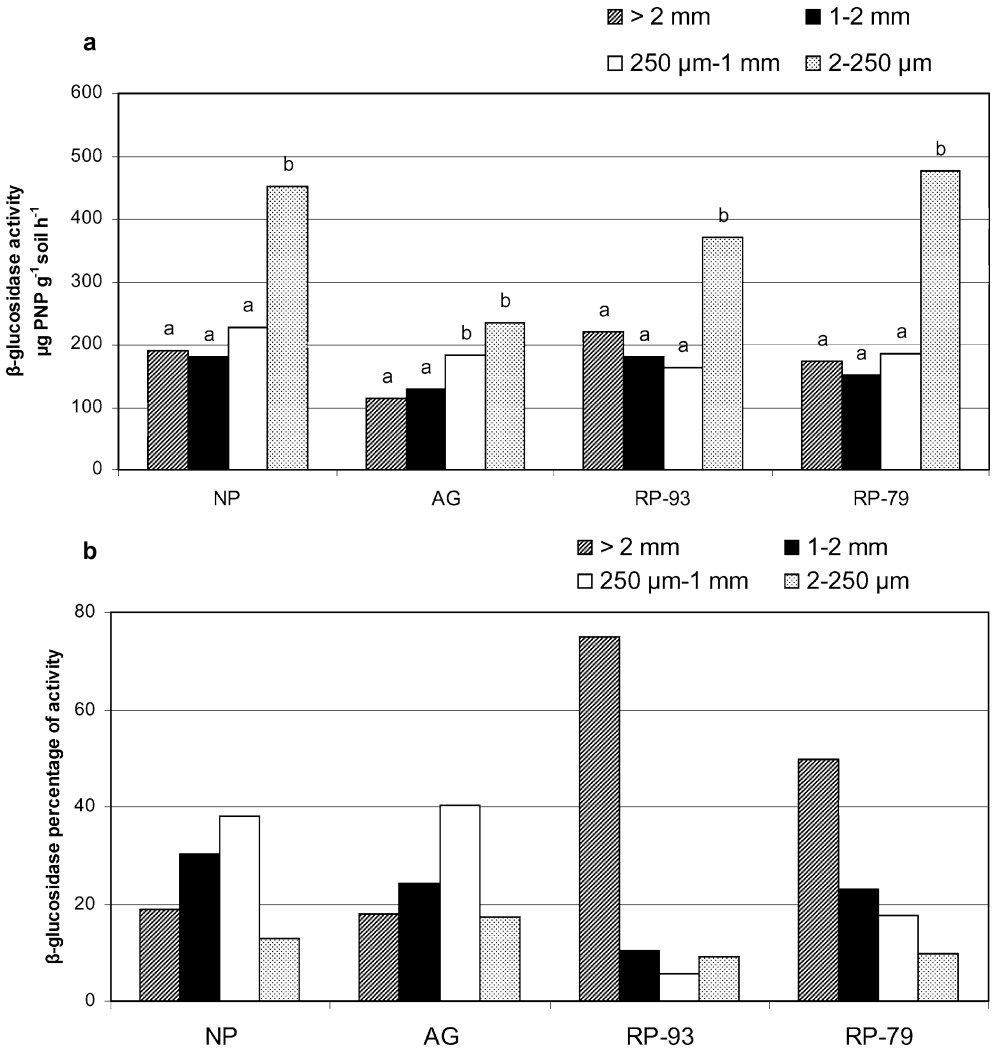

Fig. 1 **a** β-Glucosidase activity of each aggregate size fraction within each soil of the chronosequence. **b** β-Glucosidase aggregate size activity as the percent of total soil enzyme activity. *Bars* topped by the same letter are not significantly different, Bonferroni ($P<0.05$); statistical comparisons are only valid within soils (**a**)

the AG soil showed less enzyme activity across all fractions compared to enzyme activities of the other soils and aggregate fractions. In the restored grassland prairie soils (RP-79 and RP-93), β-glucosidase activity was generally greater than that of the AG soil, being significantly different in the >2 mm and 2–250 µm fractions. The NP soil only showed significantly greater β-glucosidase activity than the AG soil in the smallest aggregate size fraction (Fig. 1a).

The aggregate fraction of β-glucosidase activity as a percentage of the total soil β-glucosidase activity shows the NP and the AG soil having approximately similar activity distributions within each aggregate size fraction (Fig. 1b). Both restored prairie soils exhibited a greater percentage of their total β-glucosidase activity in the >2-mm macroaggregate size fraction than did the NP or AG soils. The contribution of the 2- to 250-µm fraction to the total β-glucosidase activity was small due to the small amount of the 2-to 250-µm fraction in each soil (Table 2). For the RP-79 soil, there was a linear decrease in the percentage from the largest to smallest aggregate size.

The amount of organic C and total N in each aggregate size fraction is shown in Table 2. That the distribution is similar to the aggregate size fraction distribution indicates there is little difference in percent organic C or total N of the fractions within soils. For each size fraction, the β-glucosidase activity was correlated with the amount of organic C (mg C g^{-1} soil), $R^2=0.72$, $P<0.001$, and also with the amount of total N (mg N g^{-1} soil), $R^2=0.67$, $P<0.001$.

N-Acetyl-β-glucosaminidase activity

The *N*-acetyl-β-glucosaminidase (NAGase) activity varied across the chronosequence soils with the NP and AG soils having even distributions of enzyme activity with generally lower enzyme activities in the AG soil (Fig. 2a). The restored prairie soils had different patterns of enzyme activity, but both had the highest activity in the smallest fraction. The linear increase in activity for the RP-79 soil was significant ($R^2=0.99$, $P<0.01$).

NAGase activity of each fraction as a percentage of the total soil NAGase activity is shown in Fig. 2b. NAGase activity of the NP soil was mainly located in the 1–2 mm and 250 µm–1 mm aggregate size fractions. The AG soil had a uniform distribution of NAGase activity, such that all four aggregate size fractions contributed approximately

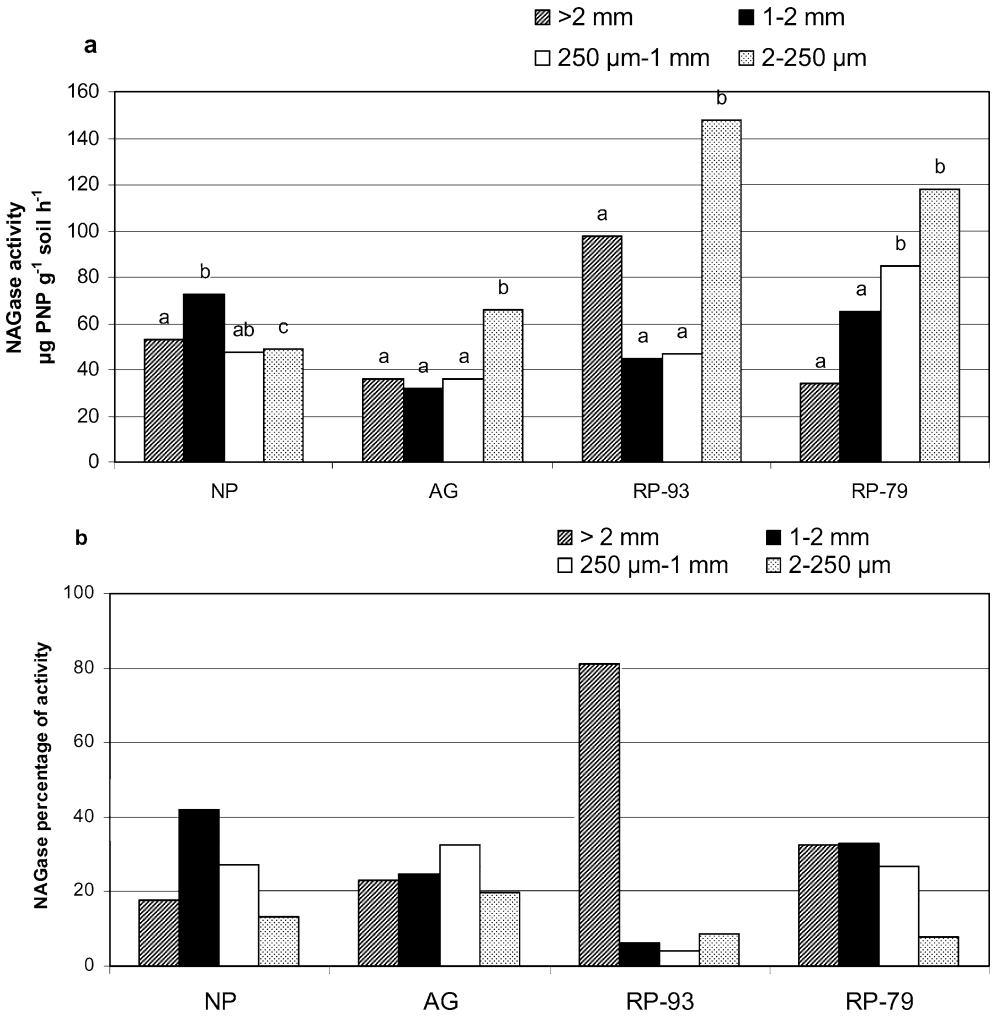

Fig. 2 **a** *N*-Acetyl-β-glucosaminidase (NAGase) activity of each aggregate size fraction within each soil of the chronosequence. **b** *N*-Acetyl-β-glucosaminidase (NAGase) aggregate size activity as the percent of total soil enzyme activity. *Bars* topped by the same letter are not significantly different, Bonferroni ($P<0.05$); statistical comparisons are only valid within soils (**a**)

equally to the total soil activity (Fig. 2b). In the RP-93 soil, the >2-mm size fraction had the greatest percentage, whereas in the RP-79 soil, the three largest size fractions had similar percentages.

For each size fraction, the NAGase activity was correlated with the amount of organic C (mg C g^{-1} soil), R^2=0.49, $P<0.002$, and also with the total N content (mg N g^{-1} soil), R^2=0.43, $P<0.006$. The low R^2 values are due to the high enzyme activity value of the >2-mm fraction of the RP-93 soil, whereas the significance of the regression reflects the close grouping of the other values.

Discussion

Soil organic matter accumulation and storage is inherently coupled to soil aggregation and facilitated by the formation and stabilization of microaggregates and macroaggregates. Current conceptual theory suggests that different binding agents are responsible for the formation of micro- and macroaggregates and that microaggregates may form within macroaggregates, further protecting and sequestering soil C (Jastrow and Miller 1998; Six et al. 2004). Breakdown or degradation of macroaggregates makes organic C in different aggregate size classes available for decomposition and loss. The compartmentalization of C and the associated enzyme–aggregate complex may determine the amount and type of C compounds that will be lost during aggregate breakdown and turnover.

The hierarchical aggregate structure proposed by Tisdall and Oades (1982) professed that microaggregates would be bound together by decomposition products into larger aggregates and eventually into macroaggregates. Elliott (1986) suggested that macroaggregates would contain more labile C that would be more readily lost upon cultivation than more resistant C associated with microaggregates. According to Six et al. (2004), based on Elliott's (1986) findings, an increase in C concentration with increasing aggregate-size class can be used to show the existence of an aggregate hierarchy in a soil. During macroaggregate turnover, C-degrading enzymes will play an important role in determining the fate of exposed labile C; thus, the enzyme–aggregate complex is essential to predicting long-term C storage.

In this study, the largest aggregate size fraction in the restored prairie soils (RP-79, RP-93) was the >2-mm fraction (Table 2). This size fraction can increase rapidly in cultivated soils that are returned to grasslands due to a rapid

increase in fine roots and fungal hyphae which promote aggregation (Jastrow and Miller 1998). The decrease in the 2-mm fraction in the prairie chronosequence from RP-93 > RP-79 > NP suggests that as soil organic matter increases and becomes more stabilized, a more even distribution of aggregate size classes is supported. In addition, in the RP soils, the C concentration of the macroaggregates increased with increasing aggregate size suggesting a hierarchical aggregate structure.

Surprisingly, the NP soil and the AG soil contained almost identical aggregate size distributions. In addition, both soils exhibited a trend of nonhierarchical aggregate structure as the C concentration decreased as the macroaggregate size increased. The similar aggregate size distributions of the NP and AG soils may result from different processes; the NP soil may be decreasing in larger size aggregates (compared to RP-79 and RP-93) due to the physical density of roots and rhizomes in the top 3–4 cm of soil which may limit space and cause large aggregate breakdown (Jastrow 1987). The aggregate distribution in the AG soil may be a result of disturbance and increased macroaggregate breakdown and/or a more even distribution of very fine roots that influence smaller macroaggregate formation (Jastrow and Miller 1998). However, both soils have greater C concentrations in the microaggregate fraction compared to the RP soils suggesting rapid macroaggregate turnover and C movement into the microaggregate fraction.

Specific enzymes cannot completely describe C and N cycling in soils since this involves complex interactions between numerous enzymes, microorganisms, and microfauna. Enzyme assays provide potential activity measurements and may have little relationship to in situ activity; however, they are useful in comparing the biochemical properties of soils with different treatments.

β-Glucosidase and *N*-acetyl-β-glucosaminidase are two important enzymes involved in C and N cycling. An advantage of using these enzymes is that they showed little change in activity due to water saturation and thus could be used in a wet sieving procedure which was necessary to quantify micro- and macroaggregates.

The greater β-glucosidase activity of the >2 mm, 1–2 mm, and 250 μm–1 mm macroaggregate size fractions of the RP-93 and RP-79 soils compared to the AG and NP soils indicates that the microbial community was likely adapting to the changes caused by the restoration. It is probable that the microbial community was responding to an increase in organic matter deposition on the soil surface due to the reduction in tillage and reversion back to a natural grassland system (Beare et al. 1992). This new input of cellulytic material would increase macroaggregate formation along with stimulation of enzyme production. This agrees with other studies (Gupta and Germida 1988; Miller and Dick 1995) that likewise concluded that macroaggregate structures provide habitat for microbial biomass and enzyme activity.

NAGase is related to both C and N cycling in soils and in the prairie soils is also indicative of fungal populations. NAGase is affected by crop rotations and fertilization but is generally correlated with total soil C and total N contents (Ekenler and Tabatabai 2002). Macroaggregate (>2 mm, 1–2 mm, 250 μm–1 mm) NAGase activity was suggestive of the compartmentalization of microbial function across the soil chronosequence. The NP, RP-79, and RP-93 soils had the highest NAGase activity in the larger aggregate fractions where the greatest concentrations of C and N reside. In addition, it is these fractions where fungal hyphae become important in aggregate formation and decomposition (Jastrow and Miller 1998). The AG soil had lower and more uniform NAGase activity in each fraction indicative of the mixing and pulverizing action of tillage (Dick 1992) and reflective of the F/B ratio. These soils could be ranked as AG > RP-93 > RP-79 by gross N mineralization rates measured by the ^{15}N isotope dilution technique (unpublished data). However, the higher activities of NAGase in the RP soils would suggest greater C and N cycling; thus, the activity within the larger aggregates must be accompanied by zones of high immobilization (Smith 1994).

As expected, the microaggregate fraction did not contribute much to the enzyme activities of the whole soils, in spite of its high activity for both enzymes, because this fraction is a very small component (4–11%) of each soil. It is possible that residual enzyme activity was protected in these microaggregates by having been once incorporated into a larger macroaggregate structure. Evidence for this conclusion is supported by $\delta^{13}C$ data, in which the age of C3 and C4 derived C in aggregate size fractions of pasture and corn soils was compared (Jastrow et al. 1996). Large macroaggregates had a greater proportion of recently deposited C and a more rapid turnover rate (74 years) than that of the microaggregate structures with a C turnover time of ~412 years. The greater amounts of labile C in macroaggregates (Elliott 1986) may be more subject to enzymatic decomposition because of the nature of the C rather than the level of enzyme activity, whereas the enzyme activity of microaggregates may be high, possibly due to immobilization of extracellular enzymes, even if the C associated with microaggregates is more resistant to decomposition.

Conclusions

The physical distribution of C-degrading enzymes is an important component of the C storage capacity of soils. In this study, we have shown that the aggregate size distribution and the distribution of the activities of two C cycle enzymes in a native prairie soil were more similar to an agricultural field than restored prairie fields. This demonstrates the complex nature of soil restoration after long-term cropping and contradicts the notion of the linear recovery of soils after disturbance. Macroaggregate formation and breakdown is important in forming soil structure; however, over time, fine roots and hyphae influence a more even distribution of macroaggregate sizes. The enzyme activity data also suggest that aggrading systems may be more vulnerable to C losses from macroaggregate turnover due to the initial rapid buildup of substrates that can be rapidly degraded by C cycle enzymes.

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